C.2.3. Amphibian Metamorphosis Assay (AMA) (OECD TG 231)

Status: Assay validated by the OECD.

311. Modality detected/endpoints: thyroid activity (advanced development; asynchronous development; delayed development in absence of non-specific systemic toxicity; thyroid histopathology), but note that this covers several different modes of action (MOA), including thyroid agonists and antagonists, as well as substances interfering with thyroid hormone synthesis and transport. According to OECD TG 231, there is disagreement about the implications of the different endpoints in this larval development screen. Some experts accept that changes in one of the thyroid-relevant apical endpoints (advanced development; asynchronous development; delayed development in absence of non-specific systemic toxicity) may on their own provide information on thyroid activity, while others will only reach this conclusion if one of the apical endpoints is accompanied by significant thyroid histopathology, such as moderate or severe follicular hypertrophy and/or hyperplasia (OECD, 2007). Note that the AMA is subject to indirect thyroid effects such as those that result from cytochrome P450 induction (e.g. phenobarbital, the model compound for the latter effect, tests positive in the AMA). Therefore, interpretation of the AMA may be complicated.

Background to the assay

312. This assay is designed as a screen for thyroid activity in amphibians, and not to provide information on endocrine activity for use in assessing the environmental risks of an individual chemical based on a predicted environmental concentration/predicted no-effect concentration (PEC/PNEC) approach. Delay in metamorphosis could be considered an apical endpoint, but the significance of a short delay in metamorphosis for amphibian populations is poorly understood except for amphibian species living in temporary pools that dry out at, or shortly after, the normal time for metamorphosis to be completed. Furthermore, the use of only three concentrations of test chemical precludes the reliable establishment of a no-observed-effect-concentration/x% effect concentration (NOEC/LOEC). It is important to note that there are several types of thyroid disruption, not all of which involve interactions with the thyroid receptor, and they have differential effects on the various endpoints in this screen. OECD TG 231 does not, however, allow unequivocal diagnosis of which type of thyroid disruption is occurring. It includes a specific endpoint (thyroid gland histopathology) for some types of thyroid activity, but also includes apical measurements (hind limb length, snout-vent length, developmental stage and wet weight), which are used to determine other thyroid-responsive endpoints: advanced development, asynchronous development or delayed development. The first two of these are considered by some authorities to be diagnostic of thyroid activity, while the latter is only diagnostic if non-specific systemic toxicity is absent. It should also be noted that a review (Pickford, 2010) concluded that for thyroid agonists, the response of amphibian thyroid histopathology is not as predictable or as sensitive as developmental stage or hind limb development. However, it is probable that a diagnosis of thyroid activity on the basis of the apical endpoints will be more robust if accompanied by thyroid histopathology, and vice versa.

313. Consequently, if the assay gives a positive result, this may be due to a combination of a positive indicator of hormonal activity (thyroid histopathology) and a positive apical endpoint (advanced development, asynchronous development or delayed development), or a positive indicator of hormonal activity alone (possibly accompanied by a negative apical endpoint), or for an apical endpoint alone (possibly accompanied by a negative indicator of hormonal activity). Each of these possible combinations of positive response should be considered separately (although the distinctions between indicators of hormonal activity and apical effects are not always clear), so they have been listed individually as points 1, 2 and 3 in the possible conclusions column of Table C.2.3. It should be noted, however, that due to the relatively short exposure time employed in this screen (three weeks), one cannot be sure if the effects of some chemicals on apical endpoints would result in adverse effects on development, growth or reproduction in the longer term. This is primarily relevant for hazard identification/characterisation. Given the high degree of endocrine system conservation across the vertebrates, endocrine-linked effects in the AMA may also indicate the possibility of related activity in other organisms such as fish, reptiles, birds or mammals.

When/why the assay may be used

314. Although OECD TG 231 could, in principle, be used at any stage in the hazard assessment process, the most likely use scenario will be when there are relatively few data available about the possible thyroid disrupting properties of a chemical. The results from this assay are most likely to be available after deployment of a battery of *in vitro* and *in vivo* screens (e.g. the United States Environmental Protection Agency's Endocrine Disrupter Screening Program), or as a supplement to existing data which suggest potential endocrine disrupter (ED) activity (e.g. a positive result in the *Xenopus* embryonic thyroid signalling assay [XETA]). A number of mammalian (rat) assays are sensitive to thyroid disruption, particularly thyroid antagonists, including the pubertal assay (male or female), the enhanced repeat dose assay (OECD TG 407) and the intact male screening assay. Note that these assays utilise different routes of exposure than OECD TG 231 and therefore, depending on the properties of the chemical, have differing potentials for the test substance to be metabolised. It should also be noted that only the AMA and the XETA appear to be sensitive to thyroid agonists. It has been argued by Pickford (2010) that only one thyroid-disrupting chemical (methoxychlor) shows activity in the AMA but not in any rodent screens, but the number of chemicals tested in the former is less than in the latter.

315. It is possible that no endocrine-relevant data are available before the AMA is deployed (i.e. if OECD TG 231 has been used as a primary screen), but in that case a positive result in the screen could be followed up with relevant *in vitro* screening to investigate the suspected MOA. However, it should be noted that *in vitro* screens essentially only exist for thyroid agonists and antagonists (e.g. GH_3 rat pituitary somatotroph cell proliferation; solid state thyroid receptor binding assays; transfected reporter gene assays in yeast or mammalian cell lines), while thyroid disruption can occur at other points in the endocrine system for which *in vitro* screens do not exist, or are still at the research stage (e.g. FRTL-5 rat cell lines sensitive to iodide uptake inhibitors) (see Paragraph 18). Furthermore, none of these screens have yet been validated and standardised at the international level.

316. In order to provide information relevant for assessing whether or not a chemical may fulfil the WHO/IPCS (2002) definition of an ED, the study design has to be sufficiently robust to demonstrate the presence or absence of effects. In the dose selection, the investigator should also consider and ensure that data generated are adequate to fulfil the regulatory

requirement across OECD countries as appropriate (e.g. hazard and risk assessment and labelling, ED assessment, etc.). The top dose or concentration should be sufficiently high to give clear systemic (i.e. non endocrine-specific) toxicity in order to ensure that a wide range of exposures (high to low) is tested. However, endocrine effects observed solely in the presence of clear systemic toxicity should be interpreted with caution and may be disregarded when sufficiently justified to be caused by secondary effects which are unlikely to be due to endocrine activity. The reason for this advice is a concern that some endocrine active substance (EAS) sensitive assays are being run at doses/concentrations of EASs that are too low to trigger direct impacts on the endocrine system. This guidance document is not the place to address this issue directly, but it should be considered when EAS-sensitive test guidelines (TGs) are revised in the future. In addition, the number and spacing of dose/concentration levels should also be adequate to fulfil the objectives of the study (e.g. to demonstrate dose response relationships if this is required).

Existing data to be considered

317. Given the commonality of endocrine mechanisms in the vertebrates, relevant existing data available before deployment of OECD TG 231 might include *in vivo* results obtained with other vertebrates (e.g. a positive *in vivo* assay with rats – see above, positive findings for thyroid endpoints in mammalian repeat dose toxicity or reproductive studies), or one or more of a range of *in silico* or *in vitro* results which suggest that thyroid disruption may occur *in vivo* (but note the limitations of this approach, as indicated above). Such indicators of possible thyroid activity might include quantitative structure activity relationship (QSAR) predictions of thyroid activity, "read-across" from *in vivo* results obtained with chemically related chemicals or positive results from an *in vitro* screen for thyroid agonist/antagonist activity. Data from the XETA, if available, should also be considered.

Scenarios: Positive and negative results combined with existing data

318. The scenarios (A to R) presented in Table C.2.3 represent all the possibilities of positive or negative results in combination with the presence or absence of existing data. The action taken will also depend on the regulatory environment, but the considerations given here are generally science based. Wherever possible, the recommended "next step which could be taken" avoids unnecessary animal testing. However, sometimes conducting an animal test will be indicated and then the relevance of species, strain and exposure route should always be considered. Further considerations specific to each scenario are given in the table.

319. Positive results obtained with the thyroid histopathology endpoint (Table C.2.3, Scenarios A-I, sub-section 2) result in the conclusion that the test chemical is a potential ED *in vivo*. If both thyroid histopathology and an apical endpoint give a response (Table C.2.3, Scenarios A-I, sub-section 1), this may provide even stronger evidence that one is dealing with a potential ED, especially if its action is not receptor-mediated. If only an apical endpoint responds (Table C.2.3, Scenarios A-I, sub-section 3), it suggests that the chemical is a possible thyroid disrupter, but with somewhat reduced confidence in some cases compared to sub-section 2 (although existing positive *in vitro* data, or positive *in vivo* data from other species, would have to be weighed against this conclusion). Note, however, that apical endpoints alone are probably sufficiently responsive to thyroid receptor agonists (i.e. in these cases thyroid histopathology is unlikely to make the assay more robust) (Daniel Pickford, pers. comm., 2010).

320. As indicated above, although a positive response of OECD TG 231 indicates that the chemical is a possible thyroid disrupter, a result of this type would generally need to be followed up with a more comprehensive growth, development and/or reproduction test if countries need further evidence (i.e. a Larval Amphibian Growth and Development Assay [LAGDA] – OECD TG 241) which is able to provide a precise NOEC/ECx for adverse effects. In other words, in order to strengthen weight of evidence, a positive result of whichever type in OECD TG 231 could be followed by a LAGDA at Level 4. Existing data suggesting endocrine-specific activity (e.g. positive *in vitro* or XETA data, or positive *in vivo* data from other species) will strengthen the case for additional testing still further. Note, however, that the LAGDA is not a true life cycle test and does not include all aspects of reproduction. For that reason, it is worth considering whether a positive result in OECD TG 231 could be more usefully followed up under some circumstances by a MEOGRT/ZEOGRT with thyroid-specific endpoints such as thyroid hormone induction or depression, although at present the responsiveness of apical endpoints in these tests (e.g. growth) to thyroid-active substances is not well understood.

321. The situation in which OECD TG 231 gives a negative result (Table C.2.3, Scenarios J-R) needs careful consideration of any existing data. If these data suggest that the chemical is endocrine active both *in vitro* and *in vivo* (Scenario J), then it is possible that OECD TG 231 is simply insufficiently sensitive, although most known thyroid disrupters have been shown to give a response in the AMA. Depending on the robustness of the existing data, it might therefore be appropriate to conduct a LAGDA.

322. If OECD TG 231 and existing *in vivo* data are all negative, but *in vitro* data reveal some endocrine activity (Scenario K), the probability is that the test chemical is not sufficiently potent to produce thyroid effects *in vivo* in amphibians or other organisms, or it may be rapidly metabolised. In such a situation, further testing is probably not necessary. However, if the chemical is known to bioaccumulate slowly, it may be that exposures in the *in vivo* tests have been insufficiently prolonged, in which case longer term testing with the LAGDA might be justified.

323. On the other hand, if OECD TG 231 and the *in vitro* tests are negative (Scenario M), but there are positive existing *in vivo* data, the nature of those existing data should be considered. Unless the existing data are from another amphibian, the chemical is probably not an ED acting on amphibian growth or development, but it may act via MOA not covered by the *in vitro* screens, or it may be more potent in species or life stages that have not been tested. In this situation, the existing *in vivo* data should be used to guide decisions about whether to conduct any further testing.

324. Finally, a negative OECD TG 231 screen, set against a background of negative *in vitro* and *in vivo* data (Scenario N), suggests that the test chemical is not a possible thyroid-active ED, and further action is unnecessary.

325. In each of the above scenarios, it is possible that existing data will be equivocal, or there may be no existing data. This will weaken the conclusions which can be drawn about a negative OECD TG 231 test, and this is reflected in Table C.2.3. However, a lack of mechanistic data on thyroid activity should ideally be rectified before any further *in vivo* testing is finally conducted, although as indicated above, many thyroid modalities are not detectable in *in vitro* screens. On the other hand, if OECD TG 231 is positive, further *in vivo* testing would generally be needed to quantify any adverse effects and/or to establish a NOEC or ECx for such effects, even if all existing data are equivocal, or if there are no existing data. Again, however, it may be useful to obtain some mechanistic information before conducting further *in vivo* testing. There is also the possibility that equivocal

mechanistic data may be the result of multiple modes of endocrine action. Under some circumstances, two opposite modes of simultaneous action (e.g. thyroid and anti-thyroid) could, depending on dose, lead to a minimisation or abolition of adverse effects, while in others two different MOA could potentially reinforce effects on certain apical endpoints. If multiple MOA are suspected, either from the existing results or based on QSAR/read-across/integrated approaches, this situation should be investigated further if needed for regulatory decision making and if necessary, the weight given to the apparently equivocal mechanistic data should be increased.

326. The scenario in which the results of OECD TG 231 are themselves equivocal has not been dealt with in Table C.2.3, for reasons of brevity. In this context, an equivocal result might be an inconsistent concentration-response (e.g. no effect at a high concentration but effects at a lower concentration) or a result which borders on statistical significance. Without knowing the exact circumstances, reliable advice cannot be given, but the opinions of an experienced ecotoxicologist should be sought. Clearly, however, such equivocal results do not necessarily rule out the existence of *in vivo* endocrine activity. For example, thyroid histopathology at a high concentration might be masked by any systemic toxicity, while growth measurements might just fail to reach a statistically significant level due to unexpectedly high variability. If these or other possible reasons for false negatives are suspected with good reason, the screen could be repeated (e.g. conduct it at lower concentrations which avoid systemic toxicity), or a more appropriate version of it (e.g. more larvae per replicate) could be designed and conducted. However, note that a repeat screen in the event of systemic toxicity would not be needed providing at least one tested concentration was not subject to such effects.

327. In summary, certain positive results in the OECD TG 231 screen may indicate that a chemical is a possible endocrine disrupter via one of several types of thyroid activity. This suggests that more comprehensive *in vivo* testing would be needed if the intention is to derive a long-term NOEC/ECx and/or to confirm whether or not the chemical is an actual endocrine disrupter due to the occurrence of adverse effects. Negative results in OECD TG 231 do not necessarily mean that the chemical is not a potential ED – a judgement about the endocrine disruption potential and the possible need for additional testing will have to be made based on a weight of evidence evaluation of existing *in vitro* and *in vivo* data.

References

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Table C.2.3. **Amphibian Metamorphosis Assay (AMA) (OECD TG 231): Guidance for scenarios of combinations of results with existing data**

This table represents possible conclusions to be drawn from assay data, and a next step which could be taken if further evidence is required about possible endocrine disrupting properties and/or effects. The guidance offered is not meant to be prescriptive, but provides science-based considerations. It encourages the use of all available data and expert judgement in a weight of evidence approach. Regional and national interpretation of results and "next steps" may vary.

The conclusions are grouped into a series of scenarios (A-R), each scenario representing a different combination of assay results, existing *in vitro* data and existing *in vivo* data. The symbol "+" indicates that the data in question represent a positive result, "-" indicates a negative result, and "Eq/0" indicates that the data are either equivocal or are not available.

Existing results: * "Mechanism (*in vitro* mechanistic data)" assumes that mechanistic data are available from thyroid hormone receptor (TR) and other assays concerning mechanisms of thyroid disruption although these are not yet in common use. In practice, data from all assays may not be available and therefore this must be taken into account when deciding on the "next step". Quantitative structure activity relationship (QSAR) predictions of TR binding/activation may be made for some substances.

Existing results: ** "Effects (*in vivo* effects of concern)" assumes effects have been observed in other *in vivo* screens/tests which give rise to concern that the test chemical may be an thyroid disrupter.

The assay under discussion could either be positive for both apical endpoints and indicators of endocrine activity, or positive just for an apical endpoint or the indicator of endocrine activity. For each scenario, each of these three possibilities is addressed separately in the possible conclusions column.

Scenarios	Result of OECD TG 231 assay (AMA)	Existing results		Possible conclusions: 1) Indicators of endocrine activity and apical endpoints positive 2) Indicators of endocrine activity positive 3) Apical endpoint positive	Next step which could be taken to strengthen weight of evidence if necessary	Other considerations
		Mechanism (*in vitro* mechanistic data)*	Effects (*in vivo* effects of concern)**			
A	+	+	+	1) Strong evidence for *in vivo* thyroid activity with potential adverse effects (developmental/growth toxicity) in amphibians, plus thyroid effects in other species. 2) Strong evidence for *in vivo* thyroid activity in amphibians, plus thyroid effects in other species. 3) Some evidence for *in vivo* thyroid activity with potential adverse effects (developmental/growth toxicity) in amphibians, plus thyroid effects in other species.	Consider performing a Larval Amphibian Growth and Development Assay (LAGDA – OECD TG 241).	Based on the limited scope of current *in vitro* screens, the positive *in vitro* data suggest that the test chemical is a thyroid (ant)agonist.
B	+	+	–	1) Strong evidence for *in vivo* thyroid activity with potential adverse effects (developmental/growth toxicity) in amphibians. 2) Strong evidence for *in vivo* thyroid activity in amphibians. 3) Some evidence for *in vivo* thyroid activity with potential adverse effects (developmental/growth toxicity) in amphibians.	Consider performing a LAGDA (OECD TG 241).	Based on the limited scope of current *in vitro* screens, the positive *in vitro* data suggest that the test chemical is a thyroid (ant)agonist. Cases where chemicals are active in the AMA but not in thyroid-responsive rodent assays are rare. In this scenario, it is therefore particularly important to discover if adverse effects appear in a longer term amphibian test.
C	+	+	Eq/0	1) Strong evidence for *in vivo* thyroid activity with potential adverse effects (developmental/growth toxicity) in amphibians. 2) Strong evidence for *in vivo* thyroid activity in amphibians. 3) Some evidence for *in vivo* thyroid activity with potential adverse effects (developmental/growth toxicity) in amphibians.	Consider performing a LAGDA (OECD TG 241).	Based on the limited scope of current *in vitro* screens, the positive *in vitro* data suggest that the test chemical is a thyroid (ant)agonist. It should be borne in mind that equivocal data may be due to a variety of causes, including experimental error, very weak endocrine activity or multiple modes of action (MOA). If the latter case is suspected, it may be necessary to investigate the matter further and/or increase the weight given to the mechanistic information.
D	+	–	+	1) Moderate evidence for *in vivo* thyroid activity with potential adverse effects (developmental/growth toxicity) in amphibians, plus thyroid effects in other species. 2) Moderate evidence for *in vivo* thyroid activity in amphibians, plus thyroid effects in other species. 3) Some evidence for *in vivo* thyroid activity with potential adverse effects (developmental/growth toxicity) in amphibians, plus thyroid effects in other species.	Consider performing a LAGDA (OECD TG 241).	The lack of *in vitro* thyroid activity is not evidence against any thyroid activity, due to the limited nature of current *in vitro* thyroid screens.

Scenarios	Result of OECD TG 231 assay (AMA)	Existing results		Possible conclusions: 1) Indicators of endocrine activity and apical endpoints positive 2) Indicators of endocrine activity positive 3) Apical endpoint positive	Next step which could be taken to strengthen weight of evidence if necessary	Other considerations
		Mechanism (*in vitro* mechanistic data)*	Effects (*in vivo* effects of concern)**			
E	+	–	–	1) Strong evidence for *in vivo* thyroid activity with potential adverse effects (developmental/growth toxicity) in amphibians. 2) Strong evidence for *in vivo* thyroid activity in amphibians. 3) Some evidence for *in vivo* thyroid activity with potential adverse effects (developmental/growth toxicity) in amphibians.	Consider performing a LAGDA (OECD TG 241).	The lack of *in vitro* thyroid activity is not evidence against any thyroid activity, due to the limited nature of current *in vitro* thyroid screens. Cases where chemicals are active in the AMA but not in thyroid-responsive rodent assays are rare. In this scenario, it is therefore particularly important to discover if adverse effects appear in a longer term amphibian test.
F	+	–	Eq/0	1) Strong evidence for *in vivo* thyroid activity with potential adverse effects (developmental/growth toxicity) in amphibians. 2) Strong evidence for *in vivo* thyroid activity in amphibians. 3) Some evidence for *in vivo* thyroid activity with potential adverse effects (developmental/growth toxicity) in amphibians.	Consider performing a LAGDA (OECD TG 241). Given the absence or equivocal nature of existing *in vivo* data, it might also be sensible to conduct a thyroid-responsive mammalian assay (e.g. rat pubertal).	The lack of *in vitro* thyroid activity is not evidence against any thyroid activity, due to the limited nature of current *in vitro* thyroid screens. It should be borne in mind that equivocal data may be due to a variety of causes, including experimental error, very weak endocrine activity or multiple MOA. If the latter case is suspected, it may be necessary to investigate the matter further and/or increase the weight given to the mechanistic information.
G	+	Eq/0	+	1) Strong evidence for *in vivo* thyroid activity with potential adverse effects (developmental/growth toxicity) in amphibians, plus thyroid effects in other species. 2) Strong evidence for *in vivo* thyroid activity in amphibians, plus thyroid effects in other species. 3) Some evidence for *in vivo* thyroid activity with potential adverse effects (developmental/growth toxicity) in amphibians, plus thyroid effects in other species.	Consider performing a LAGDA (OECD TG 241). Given the absence or equivocal nature of the *in vitro* mechanistic data, it might also be helpful to conduct an *in vitro* screen for thyroid (ant)agonistic activity.	If a new *in vitro* mechanistic assay is conducted, note that a negative resultdoes not mean that the test material has no thyroid activity. It should be borne in mind that equivocal data may be due to a variety of causes, including experimental error, very weak endocrine activity or multiple MOA. If the latter case is suspected, it may be necessary to investigate the matter further and/or increase the weight given to the mechanistic information.
H	+	Eq/0	–	1) Strong evidence for *in vivo* thyroid activity with potential adverse effects (developmental/growth toxicity) in amphibians. 2) Strong evidence for *in vivo* thyroid activity in amphibians. 3) Some evidence for *in vivo* thyroid activity with potential adverse effects (developmental/growth toxicity) in amphibians.	Consider performing a LAGDA (OECD TG 241). Given the absence or equivocal nature of the *in vitro* mechanistic data, it might also be helpful to conduct an *in vitro* screen for thyroid (ant)agonistic activity.	Cases where chemicals are active in the AMA but not in thyroid-responsive rodent assays are rare. In this scenario, it is therefore particularly important to discover if adverse effects appear in a longer term amphibian test. It should be borne in mind that equivocal data may be due to a variety of causes, including experimental error, very weak endocrine activity or multiple MOA. If the latter case is suspected, it may be necessary to investigate the matter further and/or increase the weight given to the mechanistic information.

Scenarios	Result of OECD TG 231 assay (AMA)	Existing results		Possible conclusions: 1) Indicators of endocrine activity and apical endpoints positive 2) Indicators of endocrine activity positive 3) Apical endpoint positive	Next step which could be taken to strengthen weight of evidence if necessary	Other considerations
		Mechanism (*in vitro* mechanistic data)*	Effects (*in vivo* effects of concern)**			
I	+	Eq/0	Eq/0	1) Strong evidence for *in vivo* thyroid activity with potential adverse effects (developmental/growth toxicity) in amphibians. 2) Strong evidence for *in vivo* thyroid activity in amphibians. 3) Some evidence for *in vivo* thyroid activity with potential adverse effects (developmental/growth toxicity) in amphibians.	Consider performing a LAGDA (OECD TG 241). Given the absence or equivocal nature of the *in vitro* mechanistic data, it might also be helpful to conduct an *in vitro* screen for thyroid (ant)agonistic activity. Given the absence or equivocal nature of existing *in vivo* data, it might also be sensible to conduct a thyroid-responsive mammalian assay (e.g. rat pubertal).	If a new *in vitro* mechanistic assay is conducted, note that a negative result does not mean that the test material has no thyroid activity. It should be borne in mind that equivocal data may be due to a variety of causes, including experimental error, very weak endocrine activity or multiple MOA. If the latter case is suspected, it may be necessary to investigate the matter further and/or increase the weight given to the mechanistic information.
J	–	+	+	The test chemical is probably a thyroid (ant)agonist without activity in amphibians, although it is possible that *Xenopus laevis* responds atypically in this case.	Some regulatory authorities may conclude that no further evidence is required, but it might be desirable to conduct a LAGDA with a species other than *X. laevis* (none have been validated at present) if the existing data are sufficiently persuasive.	Based on the limited scope of current *in vitro* screens, the positive *in vitro* data suggest that the test chemical is a thyroid (ant)agonist.
K	–	+	–	The test chemical is probably a thyroid (ant)agonist without activity in amphibians or other taxa, although it is possible that *Xenopus laevis* responds atypically in this case.	If there is no activity in amphibian or mammals, further evidence is probably not needed.	Based on the limited scope of current *in vitro* screens, the positive *in vitro* data suggest that the test chemical is a thyroid (ant)agonist.
L	–	+	Eq/0	The test chemical is probably a thyroid (ant)agonist without activity in amphibians, although it is possible that *Xenopus laevis* responds atypically in this case.	Some regulatory authorities may conclude that no further evidence is required, but if mammalian data are absent, it might be desirable to conduct a thyroid-responsive rodent screen (e.g. rat pubertal).	Based on the limited scope of current *in vitro* screens, the positive *in vitro* data suggest that the test chemical is a thyroid (ant)agonist. It should be borne in mind that equivocal data may be due to a variety of causes, including experimental error, very weak endocrine activity or multiple MOA. If the latter case is suspected, it may be necessary to investigate the matter further and/or increase the weight given to the mechanistic information.

Scenarios	Result of OECD TG 231 assay (AMA)	Existing results		Possible conclusions: 1) Indicators of endocrine activity and apical endpoints positive 2) Indicators of endocrine activity positive 3) Apical endpoint positive	Next step which could be taken to strengthen weight of evidence if necessary	Other considerations
		Mechanism (*in vitro* mechanistic data)*	Effects (*in vivo* effects of concern)**			
M	–	–	+	The test chemical is probably without thyroid activity in amphibians, although it is possible that *Xenopus laevis* responds atypically in this case.	Some regulatory authorities may conclude that no further evidence is required, but the positive existing *in vivo* data suggest that it might be helpful to perform a LAGDA with a species other than *X. laevis*.	The lack of *in vitro* thyroid activity is not evidence against any thyroid activity, due to the limited nature of current *in vitro* thyroid screens.
N	–	–	–	The test chemical is probably without thyroid activity in amphibians or other taxa.	No further action is necessary.	–
O	–	–	Eq/0	The test chemical is probably without thyroid activity in amphibians.	Some regulatory authorities may conclude that no further evidence is required, but if mammalian data are absent, it might be desirable to conduct a thyroid-responsive rodent screen (e.g. rat pubertal).	The lack of *in vitro* thyroid activity is not evidence against any thyroid activity, due to the limited nature of current *in vitro* thyroid screens. It should be borne in mind that equivocal data may be due to a variety of causes, including experimental error, very weak endocrine activity or multiple MOA. If the latter case is suspected, it may be necessary to investigate the matter further and/or increase the weight given to the mechanistic information.
P	–	Eq/0	+	The test chemical is probably without thyroid activity in amphibians, although it is possible that *Xenopus laevis* responds atypically in this case.	Some regulatory authorities may conclude that no further evidence is required, but the positive existing *in vivo* data suggest that it might be helpful to perform a LAGDA with a species other than *X. laevis* (none have been validated at present). Also, if clear *in vitro* mechanistic data are missing, it might be desirable to obtain some.	If a new *in vitro* mechanistic assay is conducted, note that a negative response does not mean that the test material has no thyroid activity. It should be borne in mind that equivocal data may be due to a variety of causes, including experimental error, very weak endocrine activity or multiple MOA. If the latter case is suspected, it may be necessary to investigate the matter further and/or increase the weight given to the mechanistic information.
Q	–	Eq/0	–	The test chemical is probably without thyroid activity in amphibians or other taxa.	No further action is necessary.	It should be borne in mind that equivocal data may be due to a variety of causes, including experimental error, very weak endocrine activity or multiple MOA. If the latter case is suspected, it may be necessary to investigate the matter further and/or increase the weight given to the mechanistic information.
R	–	Eq/0	Eq/0	The test chemical is probably without thyroid activity in amphibians.	Some regulatory authorities may conclude that no further evidence is required, but if mammalian data are absent, it might be desirable to conduct a thyroid-responsive rodent screen (e.g. rat pubertal).	It should be borne in mind that equivocal data may be due to a variety of causes, including experimental error, very weak endocrine activity or multiple MOA. If the latter case is suspected, it may be necessary to investigate the matter further and/or increase the weight given to the mechanistic information.